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STUDIES OF FERTILIZATION.

VIII. On the Measure of Specificity in Fertilization Between Two Associated Species of the Sea-urchin Genus Strongylocentrotus.[1]

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CONTENTS.

I. Introduction.

In previous studies on fertilization the writer has maintained that the substance from eggs that agglutinates spermatozoa of the same species is necessary for fertilization; more specifically that the activating effect of the spermatozoön in fertilization is primarily on this substance, which then sets the other processes in operation. The agglutinating substance was accordingly called

[1] The writer wishes to express his indebtedness to J. Nelson Gowanlock for his devoted and careful work as assistant in these experiments and in those of study IX.

fertilizin. If there is such a connection between the capacity of the eggs for agglutinating spermatozoa and for being fertilized, then, in addition to other consequences which have been followed out by Just (1919), Moore (1916, 1917) and the writer (1914, 1919), the fertilization reaction and sperm agglutination should show a similar degree of specificity. This has been shown to be the case in wide crosses, as between *Arbacia* and *Nereis* by the writer, and between *Arbacia* and *Echinarachnius* by Just. But the matter has not been tested with two species of a single genus where the test might be expected to be crucial. If there were no connection between agglutination of spermatozoa and fertilization of the egg, the phenomena need not exhibit similar specificities; but if the phenomena were found to be similarly specific we would have a new and strong argument for the postulated connection.

The problem of specificity in fertilization is at present obscure, and in any case data presenting the nature and degree of specificity could not fail to be useful. It is rather extraordinary that no attempt to secure even roughly quantitative results on this problem in animals has hitherto been recorded.

In the neighborhood of Pacific Grove, California, *Strongylocentrotus purpuratus* and *S. franciscanus* are found in protected situations along the rocky shores. *S. purpuratus* occurs a short distance below the high-water mark and extends to an undetermined depth, certainly several feet, below the low-water mark. *S. franciscanus* rarely occurs above the low-water mark and certainly extends into deeper water than *purpuratus*. At low water one thus finds *purpuratus* in isolated tide pools and sometimes entirely exposed, whereas *franciscanus* is very rarely found in such situations. The two species are commonly, though by no means always, intermingled just below the low water mark. In one collecting station *purpuratus* was so abundant in this zone as to form a veritable pavement on the floor and a covering on the sides of the partially open rock-pool; *S. franciscanus*, very conspicuous by its larger size, longer spines and different coloring, was interspersed among the individuals of the other species.

At the time of my visit, January and February, 1920, both species were ripe simultaneously at this station and elsewhere; but whereas all individuals of *purpuratus* had practically only

ripe gametes wherever found, *franciscanus* at this station and elsewhere, with one exception, had relatively undeveloped gonads, which, however, always contained ripe gametes, often in considerable abundance, which fertilized readily. At the exceptional station, well within Monterey Bay, all individuals of both species had perfectly developed gonads. The writer wishes to express his appreciation of the hospitality of the Hopkins Marine Station during this investigation, and his thanks to the Director, Dr. W. K. Fisher, for his aid in securing material and in many other essential ways.

II. Comparison of Gametes.

S. franciscanus is much larger than *S. purpuratus,* commonly almost twice the diameter, and its much larger spines cause the impression of an even greater discrepancy in size. It is an interesting fact that the gametes of the larger species are almost correspondingly larger than those of the smaller, both in the case of the ova and also the spermatozoa. The eggs of *franciscanus* are 110 to 114 μ in diameter, the eggs of *purpuratus* from 75 to 79 μ. The head of the spermatozoön of *franciscanus* is about 7 μ long by 2 μ broad at the base, that of *purpuratus* is about 4 by 2 μ. The jelly surrounding the *franciscanus* egg is about 30 μ in thickness and relatively firm and resistant, that surrounding the egg of *purpuratus* is about 15 μ in thickness, relatively soft and easily lost.

III. Cross-fertilization.

1. *General.*

Loeb's recorded observations contain the only information in the literature on the subject of the cross-fertilization of these two species. They are to the following effect: (1) "If we mix eggs of *franciscanus* and *purpuratus* in sea-water and add the sperm of *purpuratus* the eggs of *purpuratus* will be fertilized more quickly than the eggs of *franciscanus;* and the reverse is true if the sperm of *franciscanus* is added to a mixture of both eggs in sea-water" (p. 273, *Am. Nat.,* 1915). (2) "The sperm of *purpuratus* shows no trace of cluster formation with the egg-sea water of *franciscanus,* and yet the eggs of *franciscanus* are readily fertil-

ized with the sperm of *purpuratus*." (Loeb, 1914, p. 136.) (3) In his "Artificial Parthenogenesis and Fertilization" (The University of Chicago Press, 1913), p. 293, Loeb gives figures of plutei from *purpuratus* eggs fertilized by *franciscanus* sperm. These records of the occurrence of both reciprocal fertilizations give, however, no measure of the degree of specificity; in fact, there is only a bare hint that the straight fertilizations occur more readily than the crosses.

If one wishes to gain a correct idea of the degree of specificity in fertilization, it is necessary to control the variable factors very carefully. Assuming that only perfectly ripe gametes are used, as was the case in all the recorded experiments, the principal precautions to be observed are the following: (1) The eggs should be washed in at least two changes of sea-water to get rid of tissue secretions, blood or detritus; the eggs used should be uniform in this respect, and similar quantities should be used in comparable experiments. (2) The sperm should be sufficiently abundant so that measurable quantities of the *dry* sperm free from any fluid or foreign particles may be used as a basis for calculating the sperm concentration in any experiment. (3) The range of individual variability with reference to cross-fertilization should be understood; it is at least very considerable. (4) The method of mixing the sperm with the eggs should be as uniform as possible, or very considerable differences in percentage results may occur from this cause alone. (5) It should go without saying that the utmost precautions against contamination must be observed: abundance of sterilized pipettes and glassware, washing of specimens in drinking water to destroy adherent spermatozoa, sterilization of hands and instruments after handling any male, etc. (6) In spite of all precautions there will remain a certain degree of residual variability that shows clearly that all the conditions of fertilization are not yet understood.

Sperm Concentrations.—The basic sperm suspension from which fertilizations were usually made is one drop (0.1 c.c.) dry sperm thoroughly mixed in 5 c.c. sea-water; one drop (0.07 c.c.) of this suspension added to eggs in 100 c.c. sea-water will be arbitrarily selected as *unit sperm concentration*. Any measured insemination may be expressed as unity or as a fraction or multiple

thereof. The various inseminations are all so designated. Unit sperm concentration would usually be called a "light insemination" and ten unit concentration would usually be regarded as fairly "heavy." The absolute value of "unity" would be one part of the dry sperm in about 70,000 parts of sea-water.

There is not much difference in the relative fertility of the crosses between the two species. In the records given it appears that *franciscanus* eggs may give a small percentage of cross fertilization at lower sperm concentrations than *purpuratus* eggs; but the *purpuratus* spermatozoön is much smaller than the *franciscanus* spermatozoön, so that it is probable that sperm suspensions of *purpuratus* rated as of the same concentration as those of *franciscanus* really contain a very much greater number of spermatozoa, which would tend to explain the lower concentration for minimum results in cross fertilization of *franciscanus* eggs. On the other hand, the highest percentage of fertilizations recorded in a cross (Table 4) concerns *purpuratus* eggs; this may be due to the much greater number of experiments with this cross than with the reciprocal.

The cross-fertilized *purpuratus* eggs appear to be more viable than the cross-fertilized *franciscanus* eggs; the latter do not in my experience develop to the pluteus stage, while the former do readily enough. The blastulae even of the cross-fertilized *franciscanus* eggs ofter appear abnormal, and the gastrulae very commonly so. It is doubtful to what factor to ascribe this difference, whether to the large size of the cytoplasmic mass of the *franciscanus* egg and small size of the *purpuratus* spermatozoön, or to behavior of the chromosomes in the reciprocal crosses. Material is on hand for investigating the latter possibility.

Another difference noted in the two reciprocal crosses is that the cross-fertilized *purpuratus* eggs commonly form as fine membranes as the straight fertilized ones, whereas in a much higher percentage of *franciscanus* eggs the membranes are either "tight," in the sense that they do not stand out so far from the surface of the egg, as in the straight fertilization, or lacking entirely. Now the tight membrane and absence of membrane in fertilized eggs are signs of poor condition or low vitality. This difference,

which is far from being absolute, would appear to indicate that as a rule the *franciscanus* egg is understimulated by the *purpuratus* spermatozoön, and this may aid to explain their lesser viability as compared with the reciprocal cross.

When high sperm concentrations are used (5 units and above) the jelly of the crossed *franciscanus* eggs becomes permanently packed with *purpuratus* spermatozoa, thus producing the appearance of halos varying in intensity with the concentration of the sperm. Such halos do not appear in the reciprocal cross, nor yet in either of the straight fertilizations. The difference in the physical characters of the jelly in the two kinds of egg does not explain this difference, for the *franciscanus* sperm does not form comparable halos in *franciscanus* eggs. It would appear to be an effect of some secretion contained in the *franciscanus* jelly on the *purpuratus* sperm; but the *franciscanus* egg-water has no apparent effect on the *purpuratus* sperm.

We shall consider first the variability of different individual combinations in the two crosses separately, classified again by the sperm concentration; secondly, we shall consider the effect of various sperm concentrations on the percentages of fertilization in the eggs of one female; third, we shall tabulate the complete experiments in which both crosses and both controls were run simultaneously. The first table will give the measure of variability of different individual cross combinations. The second will give the effects of sperm concentrations for given combinations. The third will give the measure of specificity, which cannot be evaluated without the other two.

The percentages of fertilization may be measured either by the percentage of membranes formed, or by the percentage of eggs that actually segment. The latter determination is much more accurate, for membrane formation is apt to be defective in the cross-fertilized eggs, or even absent, more especially where *franciscanus* eggs are used. In some cases, both determinations were made, and the difference gives approximately the percentage of membraneless eggs that segment. Eggs that form membranes sometimes fail to segment, though this is relatively rare; it appears especially in the lower sperm concentrations.

2. *Variability of Different Individual Cross Fertilizations.*

(*a*) *Purpuratus Eggs Fertilized by Franciscanus Sperm.*—For the purpose of bringing out the individual variability of cross combinations we shall consider first the fertilizations made with sperm of 10 units concentration (Table I); temperature approximately 15° C. Each entry stands for a separate female.

TABLE I.

VARIATION OF FERTILITY OF PURPURATUS EGGS FERTILIZED WITH 10 UNITS FRANCISCANUS SPERM.

No.	Percentage of Membranes.	Percentage of Cleavage.	Remarks.
1	....	12%	One *franciscanus* male
2	1.6%	4%	One *franciscanus* male
3	1.7%	16%	
4	13 %	29%	
5	4 %	39%	
6	17 %	72%	
7	19 %	84%	
8	0	....	One *franciscanus* male
9	1 %	....	
10	1 %	....	
11	4.5%	....	
12	6.3%	....	One *franciscanus* male
13	20.1%	....	One *franciscanus* male

The variations of fertility in this cross, from 0 to 84 per cent. of cleavage, is exceeding striking and shows a great range to exist on the female side (cf. especially Nos. 2–7 where one *franciscanus* male was used). There is a probable considerable variation also on the male side as appears when the group 2–7 fertilized with one *franciscanus* male is compared with the group 8–11 fertilized with another *franciscanus* male. But no systematic attempt was made to evaluate this side, as the *franciscanus* material was relatively rare and difficult to obtain. It should also be noted that such high percentages as Nos. 6 and 7 were never approximated in any other combination (43 in all recorded and many others tried).

To correct the impression that this cross-fertilization is very easy, which might readily be given by the exceptional percentages in Nos. 2–7, the following record of the fertilization of the eggs

of five *purpuratus* with the sperm of one *franciscanus* at 13.2 units will be useful: percentages segmented 0.1 per cent., 0.3 per cent., 0.6 per cent., 1.6 per cent., 6 per cent.

It should be realized that sperm concentrations of 10 and more units are very much higher than would ordinarily be used in straight fertilizations, where 1 unit concentration is adequate to fertilize every egg.

(*b*) *Franciscanus Eggs Fertilized by purpuratus Sperm.*—The determinations for this cross are not nearly so numerous as for the reciprocal on account of the greater rarity of the material in its best condition. The eggs appear to be not quite so variable in their cross-fertilization capacity as those of *purpuratus,* but with a lower concentration of sperm they commonly show a higher percentage of cleavage after cross-fertilization than cross-fertilized *purpuratus* eggs; on the other hand, I have never secured so high a percentage with higher sperm concentrations as in exceptional lots of *purpuratus* eggs. Moreover, the cross-fertilized *franciscanus* eggs appear to be less viable than the cross-fertilized *purpuratus* eggs. These matters will be dealt with beyond. The data given in Table IV. throw some light on the question of variability.

3. *The Effect of Sperm Concentration.*

In the case of both species a higher concentration of sperm is required for any degree of cross fertilization than for practically perfect straight fertilization (see Table IV.). Beyond such minimum concentration, the result of increased sperm concentration is to increase the percentage of eggs fertilized up to a certain point; however, such increase is by no means proportional to the sperm concentration and sometimes it is very strikingly absent.

(*a*) *S. purpuratus* ♀ × *S. franciscanus* ♂.—Though the higher percentages of fertilization were never secured with sperm concentration below about 6 units, yet it must be said that the factor of individual variability of combinations is much more important than that of sperm concentration. An isolated illustration will

TABLE II.

EFFECT OF SPERM CONCENTRATION, PURPURATUS ♀ × FRANCISCANUS ♂.

Strength of Sperm.	Percentage of Membranes.	Percentage of Segmented Eggs.	Remarks.
1 unit........	2.5%	1+%	Eggs of one female
2 units........	2.5%	1+%	
8 "	<0.5%	....	Eggs of one female
32 "	<0.5%		
10 "	....	12%	Eggs of one female
20 "	....	20%	
40 "	....	25%	

prove this better perhaps than the figures given in Table II: On February 6 I tested the cross-fertilization of a given lot of *purpuratus* eggs with *franciscanus* sperm up to a concentration of 75 units with hardly any fertilization resulting at any concentration. The *purpuratus* eggs thus resistant to *franciscanus* sperm at all concentrations fertilized perfectly with *purpuratus sperm* (added later), and the *franciscanus* sperm used fertilized *franciscanus* eggs perfectly.

Another experiment in which the eggs of five *purpuratus* females were fertilized in each case with *franciscanus* sperm of 6.6 and 13.2 units will bring out once more the greater effectiveness of the individual variations than the sperm concentration:

TABLE III.

EFFECT OF SPERM CONCENTRATION ON EGGS OF FIVE FEMALES.
S. purpuratus ♀ × *S. franciscanus* ♂.

	Percentage Segmented with 6.6 Units Sperm.	Percentage Segmented with 13.2 Units Sperm.
♀ 1	4.2	6
♀ 2	0.4	1.6
♀ 3	0.9	0.6
♀ 4	0.3	0.1
♀ 5	0.3	0.3

There is a much greater difference between females 1 and 4 for instance than between the two lots of eggs of any one female.

(*b*) *S. franciscanus* ♀ × *S. purpuratus* ♂.—In the case of this cross again the individual variation outruns the effects of sperm concentration. Thus four samples of the eggs of one female fertilized with 1, 2, 4 and 8 units of the sperm of one *purpuratus*

gave 0.3 per cent., 0.6 per cent., 1.3 per cent., and 4 per cent. cleavage respectively. Two samples of the eggs of another female fertilized with 1 and 2 units of the sperm of another *purpuratus* gave in each case 5 per cent. cleavage. In a third case two samples of the eggs of one female fertilized with 8 and 32 units of *purpuratus* sperm gave about 6 and 5 per cent. membranes respectively; and in a fourth case three samples of the eggs of one female fertilized with 10, 20 and 40 units *purpuratus* sperm gave 30 per cent., 50 per cent., and 65 per cent. cleavage.

4. *The Measure of Specificity.*

From the preceding data it will be seen that the measure of specificity varies greatly with the individuals used, and this is a fact that must be born in mind in considering the following data. But however much variation there may be in the crosses, they contrast forcibly with the straight fertilizations whether they are considered individually or collectively.

The experiments tabulated below (Table IV.) were set up as follows: the usual precautions were observed for sterilization of animals, instruments, hands, glassware; each dish had a separate sterilized pipette used for it alone. The eggs of one female of each species were always washed at least twice, and an unfertilized control of each lot was kept, which never showed any membranes or segmented eggs. One hundred c.c. of sea water was placed in the bowls and about 1.5 c.c. of eggs was transferred to each, and the bowls then covered with glass plates. When the eggs were all ready for fertilization, the sperm was prepared in another room, and brought in and added as rapidly as possible; one lot of each kind of sperm thus always served for all the fertilizations of a given experiment. My assistant removed the cover, stirred in the sperm as added and replaced the cover before proceeding to the next bowl. In each experiment in the table only one male and one female of each species was used; straight fertilizations with the same male and female thus serve for control of the condition of the material used, and also for estimates as to the degree of specificity.

Table IV.

The Measure of Specificity in Crosses of Strongylocentrotus franciscanus and S. purpuratus.

M. = percentage of membranes formed.
S. = percentage of eggs segmented.

	Sperm Concentrations.	F ♀ × F ♂.		F ♀ × P ♂.		P ♀ × P ♂.		P ♀ × F ♂.	
		M.	S.	M.	S.	M.	S.	M.	S.
Exp. 1:									
a.....	0.05	73.3%		0	0	17 %		0	0
b.....	0.25	95 %		0	0	36.5%		0	0
c.....	0.5	99 %		0	<0.2%	56.5%		0	0
Exp. 2:									
a.....	0.25	100 %		4–6%		33 %		0	
b.....	0.5	100 %		4–6%		65 %		0	
c.....	1.	100 %		4–6%		75 %		<0.1%	
Exp. 3:									
a.....	1	85–90 %	80%	3%	5%	100 %	100%	1.5%	1.25%
b.....	2	85–90 %	80%	3%	5+%	100 %	100%	1.5%	1.25%
Exp. 4:									
a.....	8	100 %		5%		100 %		<0.5% <0.5%	
b.....	32			5%					
Exp. 5:									
a.....	10		100%	Mem-	30%		100%	Mem-	12%
b.....	20		100%	branes	50%		100%	branes	20%
c.....	40		100%	noted	65%		100%	noted	25%
				as				as	
				tight				good	

The above data show that the eggs of each species straight fertilize well at a sperm concentration which has no effect in cross fertilization. The fertilization is thus strikingly specific to an extent that is not realized when higher sperm concentrations are employed. If we take the lowest sperm concentration in each case in which any cross fertilization occurs, viz., 0.25 in the case of *franciscanus* eggs (Exp. 2*a*) and 1 in the case of *purpuratus* eggs (Exp. 2*c*) we can give a mathematical expression to the relative "ease" of straight and cross fertilization in a given lot of eggs. Thus in the case of *franciscanus* where 100 per cent. of the eggs fertilized with 0.25 *franciscanus* sperm we could say that cross fertilization was 20 times more "difficult" than straight fertilization. Or if we use Exp. 1*c* for comparison, cross-fertil-

ization would be reckoned 500 times more difficult than straight fertilization in the case of *franciscanus* eggs. In the case of *purpuratus* where 75 per cent. fertilized with 1 unit *purpuratus* sperm and < 0.1 per cent. fertilized with 1 unit *franciscanus* sperm we could say similarly that cross-fertilization was at least 750 times more "difficult" than straight fertilization. It is of course doubtful how much validity such a form of expression possesses, but it is probably sufficient to emphasize the enormous difference that actually exists, which a reader who has not worked with the material might otherwise fail to appreciate.

Attempts were made to overcome the very strong specificity; increase in concentration of the sperm has only a very limited effect as already noted. The influence of increase of temperature and of alkalinity of the sea water was also investigated in both species with strikingly negative results: Temperature—(1) *Purpuratus.* Samples of eggs of one female were fertilized with sperm of one male *franciscanus* (10 units strength) at 15° C., 17° C., 24° C. and 27° C. with the following percentages of eggs segmenting: 15°, 6.3 per cent.; 17°, 10.7 per cent.; 20.5°, 3.9 per cent.; 24°, 3.5 per cent.; 27°, 2.2 per cent. (2) *Franciscanus.* Samples of eggs of one female were fertilized with sperm of one male *purpuratus* (8 units strength) at 14° C., 17.5° C., 20.5° C., 27° C. with the following percentage of eggs segmenting: 14°, 3 per cent.; 17.5°, 6 per cent.; 20.5°, 11 per cent.; 27°, < 1 per cent.

Alkali.—(1) *Purpuratus.* Sample of eggs were fertilized with *franciscanus* sperm (10 units strength) in (*a*) 50 c.c. sea water + 0.4 c.c. N/10 KOH, (*b*) + 0.6 c.c. N/10 KOH, (*c*) + 0.8 c.c. N/10 KOH, (*d*) + 1 c.c. N/10 KOH, (*e*) + 1.2 c.c. N/10 KOH. In none of these was there any increase above the control, and in the last two decided decreases. (2) *Franciscanus.* A similar series fertilized with *purpuratus* sperm of 4 units concentration using NaOH instead of KOH; no fertilization occurred in any of the hyperalkaline solutions; the control showed 0.4 per cent. fertilization.

5. *Fertilization with Mixtures of Eggs and Sperm.*

Loeb has stated that if mixed eggs of the two species are fertilized separately with the two kinds of sperm the eggs of the same species as the sperm form their membranes more quickly than those of the other species. This would appear to be a promising method of measuring specificity provided that normally the membrane reaction is equally rapid in the two species. There is, however, a considerable difference in this respect: the eggs of *purpuratus* form membranes about 30 seconds after straight insemination, whereas the larger eggs of *franciscanus* require about 90 seconds (at about 17° C.). Thus when mixed eggs of the two species are fertilized with *purpuratus* sperm, the *purpuratus* membranes naturally form first; when the *franciscanus* sperm is used the question is difficult to answer decisively, because the *purpuratus* eggs that fertilize are usually so few that it is difficult to find them in the time available. My impression is that a small percentage forms membranes in about the usual time, but others more slowly so that after the *franciscanus* membranes are all formed some *purpuratus* membranes still arise. In this respect, as in others, there is great variability in the crosses.

The question involved would be more simply answered by timing the formation of membranes in straight and cross fertilizations. The latter determinations were not made systematically, but in those cases in which I have records of membrane formation of crosses taken some minutes apart, I find the percentages higher in later than in earlier determinations. Thus it would appear that while in a straight insemination membranes appear to form in most of the eggs close together in time, in cross inseminations membrane formation is spread over a longer period of time.

The above observations suggested the fertilization of mixed eggs not only with the sperm of each species separately but also with mixed sperm of the two species in order to determine whether there was any antagonism between the sperm of the two species. A rather elaborate experiment of this kind showed conclusively that no antagonism exists, such as is found sometimes between sperm suspensions of different phyla. Thus a mixture

of equal parts of both kinds of sperm (2 drops dry sperm to 5 c.c. of sea water in each case) was made one day at 11.58 A.M. and was used to fertilize mixed eggs at 12.02 P.M., at 12.45, at 1.49, at 2.43 P.M. and at 10.54 A.M. the next day. In the first fertilization practically all eggs of both kinds fertilized; in the second fertilization about 55 per cent. of the *franciscanus* eggs fertilized and 75 per cent. of the *purpuratus;* percentages were not estimated in the third and fourth fertilizations, but there was little if any falling off. In the last fertilization made about 23 hours after mixing, using the same eggs and sperm there was practically no fertilization. Controls consisting of the same mixed eggs fertilized with the same sperm samples used in the experiment, but unmixed, were run, showing approximately the same rate of falling off as the mixed sperm, during the first day. There is thus no evidence of any antagonism of sperm. On the second day the unmixed *franciscanus* sperm fertilized about 15–20 per cent. of the *franciscanus* eggs in the mixture, but the mixed *franciscanus* sperm fertilized practically none. The *purpuratus* eggs did not fertilize either with unmixed or mixed *purpuratus sperm.* There is thus an indication that the *franciscanus* sperm loses fertilizing power more rapidly in presence of *purpuratus* sperm than when separate.

IV. Cross-Agglutination.

In 1913 I described the phenomenon of agglutination of sperm suspensions of *Arbacia* by the egg-water of the same species; the phenomenon is a reversible one, the duration of which depends on the concentration of the egg-water. With stronger solutions the agglutinated masses are larger and last longer; with the lowest effective dilution the masses are microscopic in size and disperse in 5 or 6 seconds. It is possible by using a minimum quantity of water to a large bulk of eggs to secure an egg-water that will give the minimum reaction when diluted 1,200 times. The phenomenon is associated with an intense activation of the spermatozoa, due to a distinct substance, and is evidently caused by a change in the heads of the spermatozoa that renders them

sticky (adhesive), as an important factor at least. It is obvious that the reaction will detect only such changes in the individual spermatozoa as will cause them to adhere to one another; and that below this threshold the individual spermatozoa are probably affected to some extent; this consideration is important for the theoretical deductions.

I found also that the egg-water of the Annelid *Nereis* similarly agglutinates *Nereis* spermatozoa. But the agglutinating substances of *Arbacia* and of *Nereis* are without effect on the heterologous sperm. The reaction is thus specific between these forms. The egg-water of *Arbacia,* however, contains a substance toxic to the spermatozoa of *Nereis* and causing a coagulation of *Nereis* sperm suspensions that is rather deceptive at first sight. It is possible to remove this substance by treatment of the egg-water with *Nereis* sperm and leave the full complement of specific agglutinating substance, which can then be shown to be without visible action on *Nereis* sperm. The *Nereis* egg-water contains nothing that acts on *Arbacia* sperm.

Just ('19) subsequently showed that similar specific relations of agglutination obtained between *Arbacia* and *Echinarachnius:* "*Echinarachnius* egg-water activates but does not agglutinate *Arbacia* sperm. *Arbacia* egg-water agglutinates *Echinarachnius* sperm. This is a 'hetero-agglutination' by a substance in *Arbacia* egg-water separate from *Arbacia* fertilizin because it may be removed from the egg-water by dilution, by repeatedly washing the eggs and by precipitating it with *Echinarachnius* sperm. It is found in *Arbacia* blood. The microscopic appearance of this toxic heteroagglutination of *Echinarachnius* sperm is different from that of the iso-agglutination."

It was of considerable interest to discover how specific this agglutinating reaction really is. If the reaction indicates a substance contained in the eggs that is of fundamental significance in fertilization, as I believe, then it should be highly specific, like the fertilization reaction itself; and so it turns out, as the following data show.

The egg-waters of both species have strong agglutinating action, each on the sperm of its own species, which is quite similar

in all respects to agglutination in *Arbacia* previously described. Such egg-waters vary in strength according to the condition of the eggs used and the relative amounts of eggs to the sea-water. With perfectly fresh eggs added to about three times their own bulk of sea-water, the egg-water of either species may attain a strength of 640 agglutinating units, *i.e.*, such an egg-water may be diluted 640 times and still cause a minimum agglutination reaction in sperm suspensions of its own species.

The egg-waters of *S. purpuratus* are more variable in strength than those of *S. franciscanus* due to the fact that the jelly layer, which contains the larger part of the detectable agglutinating substance, is relatively very soft in *purpuratus* eggs and is rapidly lost by washing the eggs. Thus washed eggs of *purpuratus* rarely give as high an egg-water test as washed eggs of *franciscanus*.[1]

The agglutinating reaction is so prompt and unmistakable that it was really a great surprise to find that egg-waters of maximum strength may be entirely devoid of agglutinating action on the sperm of the other species, even undiluted.

The egg water of *S. franciscanus* never has the slightest agglutinating action on the sperm of *S. purpuratus*. The absence of reaction in this case was so invariable that it seems unnecessary to cite instances.

On the other hand, the egg-water of the *majority* of individuals of *purpuratus* may cause an apparent agglutination of the sperm of *franciscanus;* so that we may have the appearance of a lack of agreement between the reciprocals in this case just as we found between *Arbacia* and *Nereis,* and between *Arbacia* and *Echinarachnius* (Just). The egg-water from certain *purpuratus*

[1] I have confirmed Loeb's observation that eggs of *purpuratus* from which the jelly has been removed by acid do not produce sufficient agglutinating substance to agglutinate the spermatozoa. Such eggs are nevertheless capable of fertilization as Loeb has stated. The eggs of *franciscanus,* however, after removal of the jelly by acid continue to produce amounts of agglutinating substance sufficient to agglutinate its own spermatozoa. In this respect *franciscanus* agrees with *Arbacia* (Lillie, 1915). I should regard the result in the case of *purpuratus* as showing that an amount of fertilizin insufficient for sperm agglutination is yet adequate for fertilization; and would reject Loeb's ready interpretation that the fertilizin is not necessary for fertilization.

females has, however, not the slightest effect on *franciscanus* sperm, however concentrated it may be. (See Table VII., No. 6.) This is not at all unusual. The hetero-agglutinating action is sporadic, while the iso-agglutinating action is constant. On the assumption that the iso-agglutinating action is caused by a single substance we cannot have this substance sometimes agglutinating the sperm of *franciscanus* and sometimes not, unless we assume that the variability is in the sperm of different individuals of *franciscanus*. This cannot be the case, for the same sperm suspension of *franciscanus* may show the apparent agglutination with the egg-water of one individual of *purpuratus* and fail to show it with another equally strong in isoagglutination. I have also tried a non-heteroactive egg-water of *purpuratus* on the sperm of different individuals of *franciscanus* and found it negative consistently. It must therefore follow that the egg-waters of certain indivaduals of *purpuratus* contain a separate heteroactive substance.

This substance is apparently not a constituent of the normal blood of *purpuratus,* for the blood of *purpuratus* seems to be as indifferent a medium for the sperm of *franciscanus* as for the sperm of *purpuratus,* and the reverse is also true. In this respect it differs from the substance of *Arbacia* egg-water active on *Nereis* and *Echinarachnius.*

It should also be noted that the apparent hetero-agglutination differs in two other important respects from either iso-agglutination, (1) in the appearance of the reaction, (2) in the relation of duration of the reaction to dilution:

In iso-agglutination round solid masses of agglutinated spermatozoa form in a few seconds; these are preceded by strand-formations which rapidly contract, and the strands must be preceded by cloud formations which, however, condense so rapidly to strands as to make positive observation of these exceedingly difficult. This is true both with stronger and weaker solutions, the difference consisting in the scale of the phenomena and the rate of reversal. The action of *purpuratus* egg-water on *franciscanus* sperm never proceeds beyond the stage of dense clouds; it does not therefore resemble the action of more dilute egg-

waters on own sperm, for the cloud phase is never passed in the hetero-agglutination.

In the second place, the duration of the iso-agglutination follows a very definite rule in relation to concentration of the agglutinating substance which Richards and Woodward (1915) have formulated by saying that the efficiency of the agglutinin varies with the square root of the concentration. It is certain that the duration of the agglutination is always greatly increased with doubling of concentration, and in the lower concentrations of 1 to 4 units nearly doubled.

Table V. gives one set of results in which the egg-water of six females of *purpuratus* was tested for duration of the reaction on *franciscanus* sperm at four dilutions.

TABLE V.

DURATION OF HETEROAGGLUTINATION. EGG-WATERS OF SIX PURPURATUS FEMALES ON FRANCISCANUS SPERM AT VARIOUS DILUTIONS.

	Dilution.			
	1/1.	1/8.	1/24.	1/80.
1	10 secs.	8 secs.	neg.	neg.
2	15 "	10 "	faint	"
3	6 "	18 "	10 secs.	?
4	9 "	11 "	neg.	neg.
5	15 "	16 "	8 secs.	"
6	20 "	12 "	8 "	"

It will be seen that these figures do not follow any definite rule with reference to duration of reaction and concentration, and thus contrast in the most striking way with the iso-agglutination phenomena. This may be because the action at its optimum is so slight on the individual spermatozoa of *franciscanus* as to preclude any close agglutination. The indicator (*i.e.*, the *franciscanus* sperm suspension) may thus appear to show the same reaction at different dilutions of the egg-water.

On the assumption that we are concerned with a single heteroactive substance distinct from the true agglutinin, it should be possible to remove this substance by treating the egg-water containing it with *franciscanus* sperm without disturbing the iso-

agglutinating power of the egg-water in question. This experiment was tried twice with the general result that the action on both kinds of sperm was gradually destroyed by *franciscanus* sperm, but the heteroactive substance disappeared more rapidly. Thus no clear cut result was obtained.

The determined facts might conceivably find an explanation on the assumption that the action of *purpuratus* fertilizin on *franciscanus* sperm varies with a state of aggregation of the *purpuratus* fertilizin. I do not, however, find such an explanation satisfactory because under experimental conditions, apparently identical, certain samples of *purpuratus* fertilizin show this effect and another does not, and there is no comparable variation for the iso-agglutination.

The eggs used in the cross-agglutination experiment cited previously (Table V.) were fertilized uniformly with *franciscanus* sperm (10 units) with the following results:

Table VI.

Comparison of Cross-agglutination and Fertilization.

Purpuratus ♀.	Cross Agglutination at 1/8 Dilutions.	Fertilized with Franciscanus Sperm.	
		% Membranes.	% Cleavage.
1	8 secs.	19%	84%
2	10 "	1.7%	16%
3	18 "	17%	72%
4	11 "	13%	29%
5	16 "	1.6%	4%
6	12 "	4 %	39%

A second experiment in which the same eggs of six *purpuratus* were used for cross-agglutination and cross-fertilization gave the following results (Table VII.).

Table VII.

A Second Experiment on Cross-agglutination and Fertilization.

	Cross Agglutination.	Fertilized with *franciscanus* Sperm.
1	strong	0.3% segmented.
2	"	7.4% "
3	"	0 "
4	"	0.2% "
5	"	14.9% "
6	absent	0 "

It is thus impossible to establish any relationship between cross agglutination and cross fertilization; *i.e.*, to predict from the cross-agglutination of *franciscanus* sperm by *purpuratus* egg-water what the success of the cross-fertilization of these eggs might be. This result is to be expected from the conclusion that the apparent cross agglutination is caused by a substance distinct from the *purpuratus* fertilizin, that is, by a substance not directly concerned in fertilization.

General.

The outstanding result of the investigation is that the reaction of the egg to the spermatozoön and the reaction of the spermatozoa, by agglutination, to egg secretions are both highly specific. Fertilization and agglutination run in the same direction, and this gives strong new additional evidence that there is a specific connection between fertilization and the capacity for agglutination of the spermatozoa. It is in fact hardly conceivable that such a reciprocal specificity between egg and spermatozoön should be without significance.

Let me now deal with the apparent difficulty that agglutination (the reaction of the sperm to egg-secretions) appears to be more specific than fertilization (the reaction of the egg to the spermatozoön). As I have already pointed out in the present paper, the agglutination reaction will detect only such changes in the individual spermatozoa as will cause them to adhere to one another; any change below this threshold will fail to be distinguished. On the other hand, the fertilization reaction is to be detected in the individual egg. A higher degree of detectible specificity may therefore be expected for the agglutination reaction than for the fertilization reaction.

Secondly, the adhesion of spermatozoa to one another is no part of the process of fertilization; indeed, such adhesion could not occur in the sperm concentrations ordinarily used in fertilization, or occurring in nature, owing to the wide dispersion of the spermatozoa. It is the change in the individual spermatozoön and the postulated reciprocal change in the fertilizin that is

significant, not the adhesion of spermatozoa to one another that results in sufficiently concentrated sperm suspensions.

One cannot therefore argue from the absence of the agglutination reaction in any given case that there is no connection between the modification of the spermatozoön evidenced by agglutination and fertilization. This is admittedly a serious limitation of the method for analysis of fertilization, but the limitation does not weaken the force of the positive results.

My previous interpretation of the phenomena of fertilization was that the substance that agglutinates the spermatozoa is the same as that which activates the egg. I therefore named this substance fertilizin, for its presence is essential for fertilization. The fertilization reaction was thus conceived to be a combination of a sperm component ("sperm receptors") with the fertilizin of the egg, and a consequent activation of the fertilizin which then reacts with egg components ("egg receptors") initiating the developmental processes.

This conception furnishes a schema within which the complex events and immediate consequences of fertilization can be readily comprised. The original arguments (Lillie, 1914) for the necessity of fertilizin in the fertilization reaction were as follows: (1) The production of fertilizin by eggs ceases at fertilization; correspondingly the eggs are "immune" to spermatozoa. (2) The production of fertilizin by eggs ceases after membrane formation by butyric acid; correspondingly these eggs are not fertilizable. (3) If the fertilizin content of eggs be reduced by repeated washings the fertilization capacity decreases correspondingly. (4) The production of fertilizin by eggs of the sea-urchin does not begin until after maturation; correspondingly the fully grown ovocytes with intact germinal vesicle are immune to spermatozoa, or, if spermatozoa enter such eggs, they are entirely without effect.

These results have been confirmed and extended by Just (1919) and by Moore (1916, 1917). The latter author made a special study of the butyric acid reaction with special reference to the question of superposition of fertilization; while Just has dealt with all of these points, especially in the case of *Echin-*

arachnius, and has shown a constant relationship of a very definite sort between fertilizin content of the eggs as shown by the agglutination reaction and their capacity for fertilization.

The measurements of specificity contained in the present paper are interpretable in the same way. One cannot regard the specificity in fertilization and in sperm agglutination as unrelated phenomena. The change in the spermatozoön produced by the fertilizin of its own species must in some way be connected with the relative ease with which the spermatozoön fertilizes the egg of its own species. The fertilizin theory gives a reasonable view of this relationship. The variable percentages of cross-fertilization would show that a degree of reaction which would not suffice for agglutionation of the spermatozoa may yet be sufficient under favorable circumstances for activation of fertilizin.

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